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EVALUATION OF GREEN TURTLE FIBROPAPILLOMA FOR VIRUSES

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ADMINISTRATIVE REPORT H-92-09C

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PREFACE

Prepared under contract as a cooperative effort between the Southwest Fisheries Science Center (Honolulu Laboratory) and the Southeast Fisheries Science Center (Miami Laboratory), this report provides an increased understanding of viruses associated with tumors (fibropapillomas) in the green turtle, *Chelonia mydas*. The incidence of these life-threatening tumors on green turtles in the Hawaiian Islands has increased to epidemic proportions during recent years. A similar situation exists among green turtles at certain sites in Florida, the Caribbean, and other selected locations worldwide. The cause of fibropapillomas in green turtles remains unknown. The impact of the disease on the afflicted populations can have serious consequences. The disease represents one more threat to the survival of the green turtle. The nature of this disease and its cause must be determined in order to develop a long-term disease management program. The present report by Dr. Elliott Jacobson constitutes progress in that direction which must be followed up with additional studies.

Because this report was prepared by an independent investigator, its statements, findings, conclusions and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

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June 1992

INTRODUCTION

Cutaneous fibropapillomas in the green turtle (GTF), *Chelonia mydas*, were first described in the late 1930s from the Florida Keys (Lucke 1938; Smith and Coates 1938). Over the next 40 years little additional information was published on this disease. Starting in 1982, cutaneous fibropapillomas were seen in green turtles in the Indian River Lagoon System in east central Florida, and between 1985 and 1986, 57% of green turtles collected at this location were affected (Ehrhart et al. 1991). During the 1980s there was increased prevalence of GTF at a number of locations in the world including the Florida Keys (Teas 1991) and certain sites in Hawaii (Balazs 1991). GTF has also been seen at near shore sites in: Puerto Rico, Cayman Islands, Virgin Islands, Barbados, Venezuela, Colombia, Panama, Belize, and Australia.

Both light and electron microscopic characteristics of GTF have been described (Jacobson et al. 1989). In what were interpreted as the earliest lesions, there was ballooning degeneration of basal epidermal cells, with intracytoplasmic vacuoles occasionally containing particles with electron-dense centers. While these particles resembled viral particles, their exact nature could not be determined. In a recent report (Jacobson et al. 1991), two juvenile green turtles collected in the vicinity of Key West, Florida, were found to have fibropapillomas containing intranuclear inclusions within ballooning epidermal cells. By electron microscopy these particles were compatible with those of the family Herpetoviridae. It is unknown whether or not the herpesvirus identified in GTF is the etiologic agent of this disease. In order to establish a causal relationship and thus fulfill Koch's postulates, the identified virus needs to be isolated and transmission studies conducted in naive green turtles.

The objective of this study was to identify virus in fibropapillomas of the green turtle so that isolation attempts could be initiated. Multiple cutaneous fibropapillomas were collected from green turtles in Hawaii and the Florida Keys for histopathological examination. In addition to conventional histology, immunofluorescence was also performed. Finally, both cutaneous and visceral tumors were examined by flow cytometry in order to determine the DNA content of these lesions. In this report the findings of these studies are presented.

MATERIALS AND METHODS

Turtles

Biopsy specimens of multiple cutaneous fibropapillomas were collected from 7 Hawaiian green turtles in Kaneohe Bay, Oahu, Hawaii. Each tumor was sectioned into 3 portions and was processed as follows: 1) one portion was fixed in neutral buffered 10% formalin for histology, 2) one portion was placed on saline soaked gauze for flow cytometry, and 3) one portion was placed in a tube and frozen at -70°C for future viral isolation attempts by Dr. Gail Scherba, University of Illinois. From each turtle, a blood sample was obtained and placed in a lithium heparin microtainer tube and solid tissues were collected in either saline soaked gauze or in cryotubes and shipped on ice packs. Upon receipt all samples were immediately frozen at -80°C.

Cells harvested from the dermis of normal skin and cutaneous fibropapillomas and from the core of visceral nodules were obtained by scraping the frozen samples for three to five minutes with a #10 scalpel blade into 1 ml aliquots of Hanks buffer. Cell concentrations were measured on a hemocytometer using 30 μl of cells from solid tissues which were stained with 70 μl of a propidium iodide based fluorescent solution. For blood samples, 10 μl of cells were stained with 90 μl propidium iodide solution. The concentrations were adjusted to between 500,000 and 2.5 million cells per ml. Each sample of cells was then centrifuged (200 to 300 g's) in a 1.5 ml microcentrifuge tube. The supernatant was aspirated in a manner to prevent exposure of cells to the air. Following resuspension with 1 ml of Hanks buffer, the samples were filtered through 40 um nylon mesh to remove the visible clumps, followed by slow centrifugation at 200-300 g's. Next the samples were resuspended in 50-100 μl of Hanks buffer. Samples were then processed with a Becton Dickinson Cycle Test DNA Reagent Kit which follows the Vindelov method of cell dissociation with trypsin and fluorescent staining with propidium iodide. A FACSCAN (Becton Dickinson) flow cytometer was calibrated with the nucleated blood from both chickens and from green sea turtles which had been judged as normal, healthy animals. The FACSCAN was programmed to collect 20,000 cells per sample. The resulting histograms were analyzed on a Hewlett Packard computer which was programmed to operate and support the FACSCAN system.

RESULTS

Histological Evaluations and Immunofluorescence

From 5 to 12 biopsies of fibropapillomas were examined from each of 7 Hawaiian green turtles. By light microscopy the surface of many of these biopsies were thrown into low to high

papillary projections, supported by broad fibrovascular stalks. In many tumors, the epidermis was mildly hyperplastic and rete pegs extended into the dermis. The dermis ranged from being hypercellular to primarily collagenous. Most biopsy specimens in 5 of the 7 turtles sampled had mild to moderate amounts of inflammatory cell infiltrate around vessels in the dermis. In 8 of 12 biopsies from one turtle and all 5 biopsies from a second turtle, there was no inflammatory cell infiltrate in the dermis. In biopsies of 6 of 7 turtles sampled there were from 1 to 12 trematode eggs seen within a 7 μm section of examined tumor. Trematode eggs were absent in each of 5 biopsies in one turtle. There appeared to be no correlation between the degree of inflammation around dermal capillaries and the number of eggs seen within capillaries.

From 1 to 11 fibropapillomas were examined from each of 12 green turtles from the Florida Keys. By light microscopy, except for a few notable exceptions, the fibropapillomas were of similar morphology to those from Hawaiian green turtles. Overall there were fewer trematode eggs within dermal capillaries compared to the Hawaiian biopsy specimens. Only 2 of the 12 turtles examined had trematode eggs within dermal capillaries. In 3 necropsied turtles, although no eggs were seen in cutaneous fibropapillomas from these turtles, the spleens contained from a few to moderate numbers of trematode eggs. Inflammatory cell infiltrates were seen in the dermis, particularly around capillaries, even though few in the cutaneous fibropapillomas.

In one fibropapilloma from a Florida green turtle, there was ballooning degeneration in a solitary focus of epidermal cells with acidophilic staining intranuclear inclusions. These inclusions were consistent with those reported from green turtles from the Florida Keys which were composed of herpesvirus (Jacobson et al. 1991).

Flow Cytometry

All blood samples from both clinically normal green turtles and green turtles with fibropapillomas from Florida and Hawaii produced histograms with normal diploid profiles (Figures 1 and 2). Coefficients of variation (CV) ranged between 2.2% and 6.8%, with a mean of 3.6%. Some variability in the quality of samples shipped to us, i.e., partial coagulation, freezing artifact, etc., may account for several high CV's. Normal skin samples, fibropapillomas and visceral tumor nodules also yielded histograms of normal DNA content (Figures 3,4,5 and 6). CVs for these samples ranged between 2.5% and 9.5%.

DISCUSSION

The pathogen(s) or environmental insult responsible for the development of fibropapillomas among green sea turtles has not

yet been determined. Recently, Jacobson et al. (1991) reported on the presence of a herpesvirus in two cases of GTF from the Florida Keys. While herpesviruses have been associated with and/or demonstrated to be the cause of neoplastic diseases of a variety of vertebrates including renal adenocarcinoma of the leopard frog (Lucke 1952), papillomas in the European green lizard (Raynaud and Adrian 1976), Marek's disease of poultry (Powell 1985), papillomas in the African elephant (Jacobson et al. 1986), and Burkitt's lymphoma of humans (Werner and Gertrude 1982), there are still examples of probable secondary infection of tumors such as in bovine ocular squamous cell carcinoma (Anson et al. 1982). In order to fulfill Koch's postulates and determine if a causal relationship exists between the herpesvirus demonstrated in fibropapillomas and GTF, multiple biopsies of tumors from green turtles in Hawaii and the Florida Keys were examined in an attempt to identify cases of herpesvirus infection. Of the 73 fibropapillomas examined from Hawaiian green turtles, evidence of herpesvirus infection, such as formation of intranuclear inclusions, were not observed in any of the sections examined. Of the 68 fibropapillomas examined from affected green turtles in Florida, only 1 was found to have intranuclear inclusions. A portion of this tumor was delivered to Dr. Gail Scherba, at the University of Illinois, for viral isolation attempts. To date, tissue culture infection and virus propagation have been unsuccessful. However, the turtle from which this tumor was biopsied remains alive in captivity in Marathon, Florida, and will be used for further studies.

Trematodes of the family Spirorchiidae, which are commonly encountered in the cardiovascular system of marine turtles, were seen more often within dermal capillaries of cutaneous fibropapillomas of green turtles in Hawaii compared to Florida. While trematode eggs were in moderate numbers in the spleens of necropsied Florida green turtles, few eggs were seen in sections of fibropapillomas from the same turtles. Still, it may take multiple sections of a given tumor to clearly demonstrate the presence or absence of eggs. The role of these parasite eggs in GTF, if any, is yet to be determined.

A preliminary effort was made to identify antigens that are specific to green turtle fibropapilloma using indirect immunofluorescence tests. A mouse monoclonal antibody directed against the immunoglobulin light chain of desert tortoise, and cross reactive with green turtle light chain, was used in these tests. The results of these experiments suggested that no anti-tumor antibodies were made by the affected turtle evaluated. However, this interpretation was based on the immune response of only one turtle. Many samples will have to be examined before this approach can be abandoned. Specifically, it is critical to test sera from green turtles that have recovered from GTF (our turtle was assumed to be recovering based on the size and shape of tumors, however the history was too incomplete to distinguish tumor regression from stasis or slow progression). The results

obtained in this portion of the study have at least shown that direct and indirect immunofluorescence is feasible with this tumor since background non-specific fluorescence could be attenuated with appropriate dilution of reagents. Since the tumor containing herpesvirus-like inclusions was from a green turtle that is currently alive in captivity in Marathon, Florida, serum samples will be collected from this turtle for further immunofluorescence studies.

Several fibropapillomas from both Florida and Hawaiian green turtles were examined by flow cytometry to determine the DNA content of these tumors. Additionally, several visceral tumor nodules obtained at the time of necropsy from Florida green turtle tumors were also examined. To serve as controls, blood samples and skin biopsies of clinically normal green turtles were similarly examined. Results of this study indicated similar histograms between all samples examined. Blood from normal green turtles, normal skin from normal green turtles, normal skin from affected green turtles, cutaneous fibropapillomas and visceral tumor nodules had histograms typical of a normal diploid cell cycle. This supports the interpretation that GTF is a multicentric disease, with the agent causing proliferative growths at multiple tissue sites.

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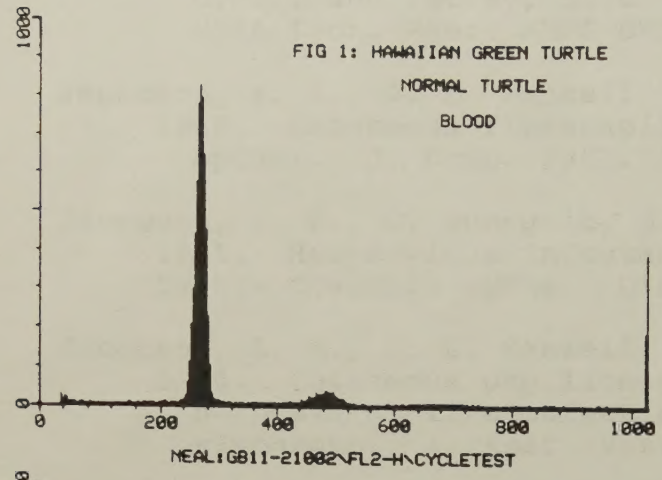

FIG 1: HAWAIIAN GREEN TURTLE
NORMAL TURTLE
BLOOD

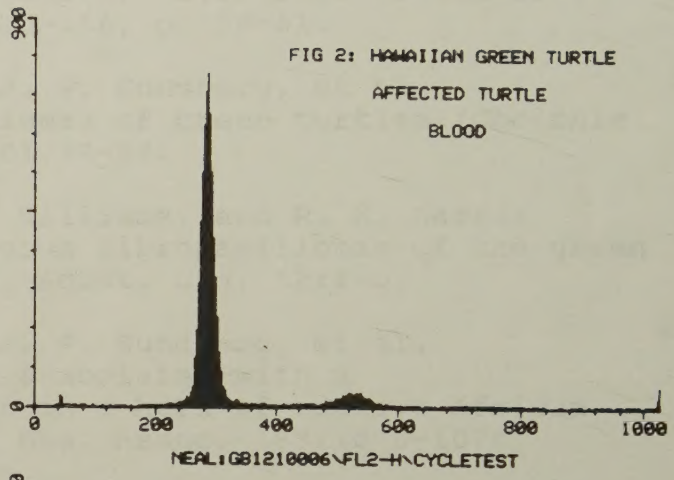

FIG 2: HAWAIIAN GREEN TURTLE
AFFECTED TURTLE
BLOOD

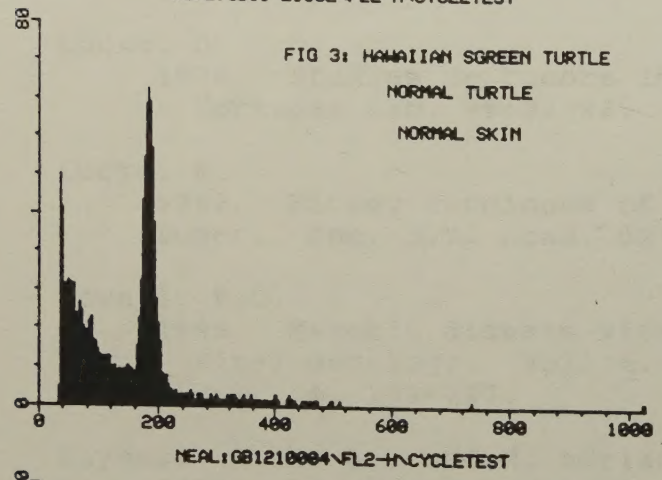

FIG 3: HAWAIIAN SGREEN TURTLE
NORMAL TURTLE
NORMAL SKIN

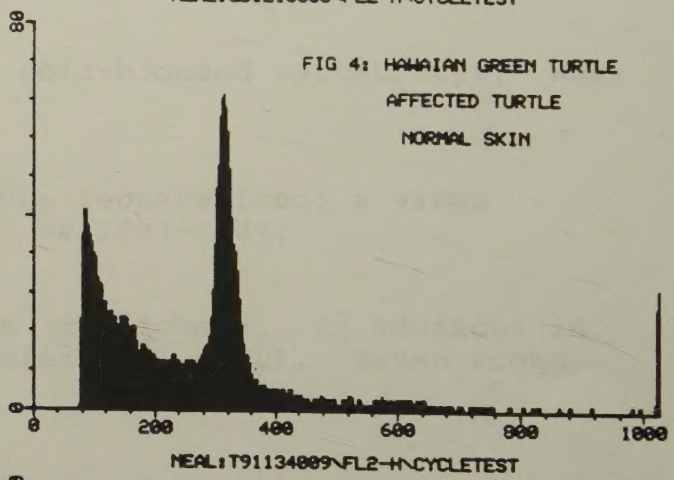

FIG 4: HAWAIAN GREEN TURTLE
AFFECTED TURTLE
NORMAL SKIN

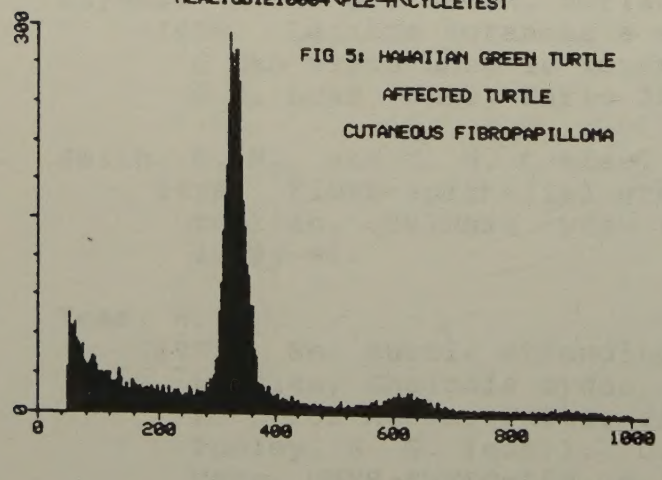

FIG 5: HAWAIIAN GREEN TURTLE
AFFECTED TURTLE
CUTANEOUS FIBROPAPILLOMA

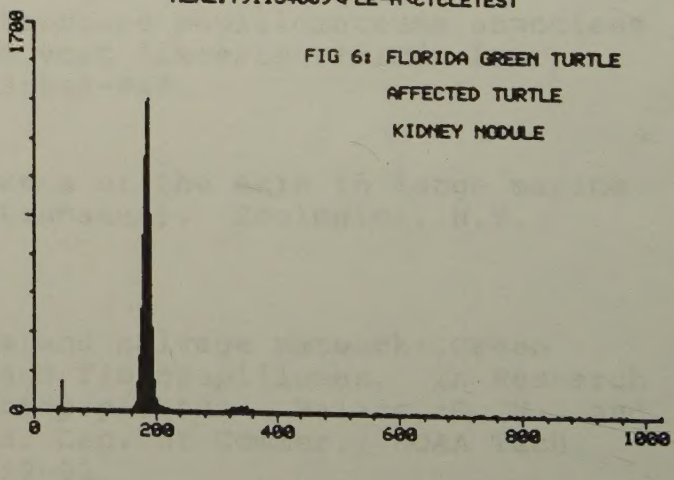

FIG 6: FLORIDA GREEN TURTLE
AFFECTED TURTLE
KIDNEY NODULE